Improved reproducibility of enrichment and site-assignment of biotinylated peptides using new anti-biotin antibody and its application to investigate redox signaling

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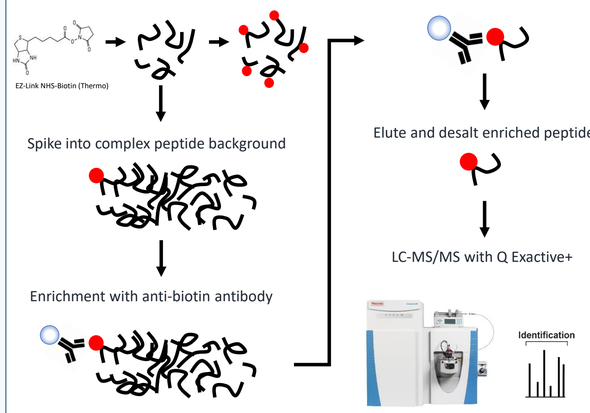
Abstract

Affinity purification of biotinylated proteins with a standard streptavidin-based enrichment is a powerful tool, but it is limited in its ability to provide site-specific information due to difficult recovery of biotin-modified peptides. Previous studies have shown that enrichment of biotin-modified peptides with an anti-biotin antibody allows for large-scale identification of biotinylated sites by tandem mass spectrometry, making it a potentially useful tool for the study of various post-translational modifications. Application of antibody-based methods to broadly purposed enrichment strategies necessitates interbatch reproducibility of antibody, leading us to test a new monoclonal anti-biotin antibody from Cell Signaling Technology. Here, we evaluate the depth and reproducibility of enrichment of biotinylated peptides using the CST antibody and compare the results to those obtained using the prior ImmuneChem antibody. We then utilize the new antibody to investigate the prevalence and potential biological roles of redox signaling in immune cells. The ability of this new antibody to purify biotin-labeled peptides will contribute to the development of robust strategies to study post-translational modifications (PTMs) and their biological implications.

Methods



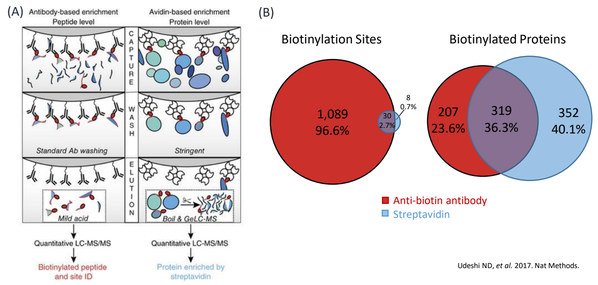
Anti-biotin immunoaffinity purification protocol

Antibody handling steps provided by Cell Signaling Technologies

1. Enrichment is from 1 mg trypsin-digested Jurkat lysate spiked with biotinylated peptide standard at a 1:1000 ratio and reconstituted in 1.5 ml IAP buffer (50 mM MOPS (pH 7.2), 10 mM Na_2HPO_4, 50 mM NaCl).
2. Incubate sample with antibody beads (PTMscan® Anti-Biotin Kit #41343) for 2 h at 4 °C, rotating end over end.
3. Save flowthrough and wash beads 2x with 1 ml cold IAP buffer.
4. Wash beads 3x with 1 ml cold HPLC-grade H_2O.
5. Elute bound peptides at RT with 55 µl 80% MeCN/0.1% TFA.
6. Repeat elution with 50 µl 80% MeCN/0.1% TFA.
7. Freeze and dry down eluted peptides in a vacuum centrifuge.
8. Reconstitute peptides in 100 µl 0.15% TFA and desalt on a two-disc C18 stage tip.
9. Perform LC-MS/MS with Easy-nLC 1000 and Q Exactive+ mass spectrometer (Thermo Fisher Scientific).
10. Search and summarize raw data with Spectrum Mill (Agilent Technologies).

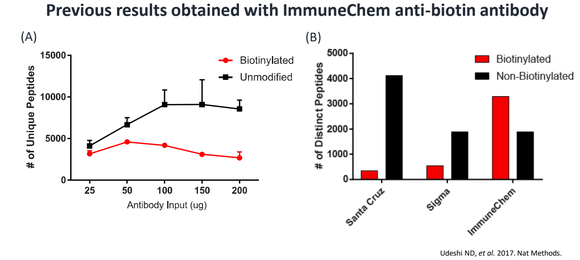
Background

Antibody- vs. streptavidin-based enrichment of biotinylated peptides



(A) Strategies for the enrichment of biotinylated peptides differ between antibody- and streptavidin-based methods. (B) In an APEX experiment, use of an anti-biotin antibody yielded significantly more site identifications and comparable protein identifications compared to streptavidin [1].

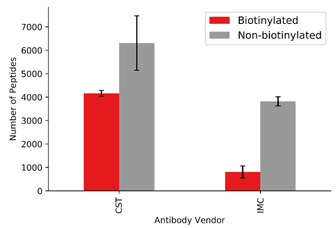
Previous results obtained with ImmuneChem anti-biotin antibody



(A) Peptide recovery from 1 mg trypsin-digested Jurkat lysate spiked with 1 µg of biotinylated peptide standard mixture enriched with varying amounts of antibody reveals an optimal antibody input of 50 µg for the ImmuneChem product [1]. (B) Comparison of biotinylated peptide yield across antibody vendors identifies ImmuneChem as the most effective [1].

Results

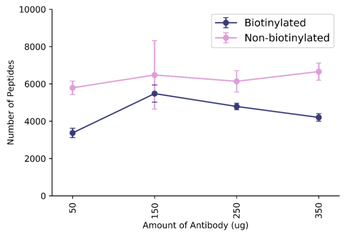
CST antibody shows improved performance compared to ImmuneChem



Vendor	Biotinylated Peptides	Enrichment Specificity (%)
CST	4157	40.0
ImmuneChem	806	17.2

The monoclonal CST antibody outperformed the current formulation of the polyclonal ImmuneChem antibody in both number of biotinylated peptide identifications and enrichment specificity. Enrichment was performed as described above for the CST antibody and as previously described for the ImmuneChem antibody [1].

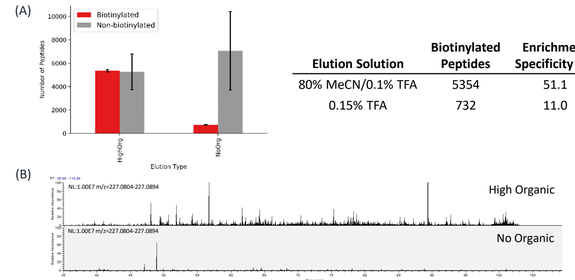
Determining optimal amount of antibody per IP



Antibody Amount (µg)	Biotinylated Peptides	Enrichment Specificity (%)
50	3370	36.8
150	5479	46.4
250	4789	43.9
350	4202	38.7

IP of biotinylated peptides was performed from 1 mg sample input using varied amounts of antibody based on the given concentration of antibody on bead (350 µg antibody per 40 µl beads). Enrichment with 150 µg anti-biotin antibody yielded the highest amount of biotinylated peptides with the highest specificity. Plotted are the mean values obtained from three replicates, with standard deviations reflected as error bars.

High organic elution increases recovery of biotinylated peptides

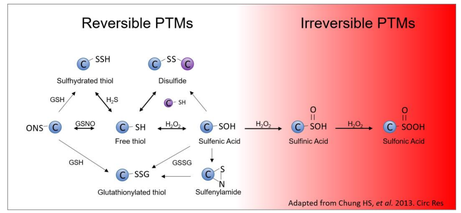


Elution Solution	Biotinylated Peptides	Enrichment Specificity (%)
80% MeCN/0.1% TFA	5354	51.1
0.15% TFA	732	11.0

(A) Elution with a high organic solution showed a higher recovery of biotinylated peptides compared to standard TFA elution, likely due to the improved ability to solubilize hydrophobic biotinylated peptides. (B) Extracted ion chromatograms at biotin fragment m/z for high organic (top) and no organic (bottom) elution.

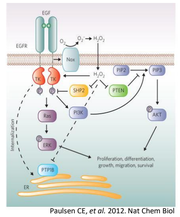
Application

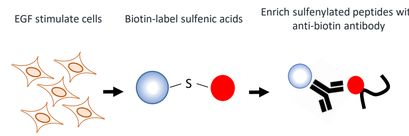
Cysteine modifications have potential implications in redox signaling



Cysteine modification is dependent on redox environment, with sulfenylation being the final step before cycling back through reversible modifications or becoming permanently oxidized. Sulfenylation is of interest to us because of its potential as a redox modulator and cross-talk with other modifications.

Cysteine sulfenylation shown to be involved in EGFR pathways



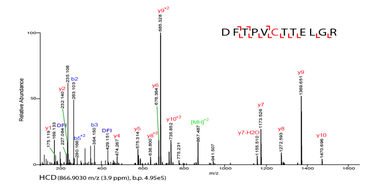


Stimulated EGFR triggers well-known phosphorylation cascade, as well as regulatory sulfenylation via Nox protein [4]. We utilize EGF-stimulation system to develop strategy for the enrichment of sulfenylated peptides.

Anti-biotin antibody successfully enriches sulfenylated peptides

Stimulation Condition	Sulfenylated Cysteines	Enrichment Specificity (%)
+EGF	524	6.35
-EGF	477	5.34

PRDX6:



High number of sulfenylated peptides identified from 1 mg input compared to benchmark (~1,000 sites from 30 mg input)[5]. Accurate identification of previously validated sulfenylation site on peroxiredoxin-6 further supports our enrichment strategy [5].

Conclusions and Future Directions

- ImmuneChem anti-biotin antibody suffers from batch-to-batch irreproducibility.
- Monoclonal anti-biotin antibody from Cell Signaling Technology reproducibly yields substantial recovery of biotinylated peptides.
- Optimal conditions for CST antibody performance include 150 µg antibody per IP (for 1 mg input) and elution in 80% MeCN/0.1% TFA.
- CST anti-biotin antibody appears to be a promising tool for the enrichment of sulfenylated peptides.

- Test high organic elution method for improved performance of other IP types.
- Continue to develop quantitative approaches to study sulfenylation in a redox signaling model.

References

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